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Synthesis of part of a mouse immunoglobulin light chain in a bacterial clone

O.Amster*, D.Salomon*, O.Zemel*, A.Zamir*, E.P.Zeelon†, F.Kantor† and I.Schechter†

Departments of Biochemistry* and Chemical Immunology†, Weizmann Institute of Science, Rehovot, Israel

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ABSTRACT

We have cloned double stranded cDNA sequences encoding a mouse immunoglobulin light chain (L-321) into the PstI site of the β-lactamase gene of plasmid pBR322 by the oligo (dG)-oligo (dC) tailing procedure. Escherichia coli X1776 transformed by the recombinant plasmids were screened for the expression of L-321 antigenic determinants by a newly developed in situ radioimmunoassay. One out of seven transformants screened was found to synthesize an L-chain like protein. Each bacterial cell produces about 550 molecules of the L-chain sequence. Preferential segregation of the L-chain sequence into the periplasmic space suggests covalent attachment of the L-chain sequence to the N-terminal portion of β-lactamase. Restriction mapping of the plasmid DNA isolated from the positive clone indicated the presence of a DNA sequence coding for the entire constant region and extending into the variable region for a length corresponding to about 40 amino acid residues. The orientation of the cloned cDNA with respect to the plasmid DNA is compatible with the formation of a fused β-lactamase-L-321 peptide.

INTRODUCTION

Since the first successful trials of insertion and propagation of eukaryotic DNA in bacteria, attention has been drawn to the potential value of such heterologous genetic systems in studies of gene expression, as well as in the synthesis of useful proteins of higher eukaryotes in bacterial cells. Early experiments have demonstrated the apparently autonomous expression of cloned genes from the low eukaryotes yeast (1,2) and Neurospora (3) to form gene products functional in bacterial cells. However, not until recently were there any indications for the expression of cloned DNA from higher organisms in bacteria. The general method applied in recent studies to promote expression is based on the integration of the foreign DNA within a bacterial gene in the cloning vehicle, anticipating transcription and in phase translation of the inserted DNA to occur by a readthrough mechanism. The expected outcome is a fused polypeptide where the amino acid sequence encoded by the foreign DNA is joined to a bacterial polypeptide. Following this general

scheme, several translational products typical of higher eukaryotes e.g., somatostatin (4), ovalbumin (5,6), rat growth hormone (7), insulin (8,9) and hepatitis virus B antigen (10), were detected in bacterial clones of the corresponding enzymatically or chemically synthesized cDNA's, or genomic DNA.

Using a similar approach we have now obtained evidence for the expression of a cloned cDNA for an immunoglobulin (Ig) κ light chain from the mouse myeloma MOPC 321 (L-321). The cDNA was inserted within the β-lactamase gene of plasmid pBR322 by the oligo (dG)-oligo (dC) joining procedure, and transformed E. coli clones were screened for the presence of L-321 antigenic determinants by a newly developed in situ colony radioimmunoassay. One positive clone was identified and further characterized. It presumably synthesizes part of the variable region and all of the constant region of the Ig light chain. These results indicate the possibility of the synthesis of specific antibody molecules in bacterial cells.

MATERIALS AND METHODS

mRNA, cDNA and dsDNA. L-321 mRNA was prepared from MOPC-321 myeloma polysomes specifically precipitated by antibodies to the L-chain and further purified by chromatography on oligo(dT)-cellulose (11). The purity of the L-321 mRNA obtained was estimated to be over 95% by several independent criteria (12, 13). This mRNA served as template for AMV reverse transcriptase to prepare the cDNA (14) which was then converted to the double stranded (ds) DNA using the same enzyme (15).

Construction of chimeric plasmids, transformation and identification of clones. The dsDNA was digested with S1 nuclease and deoxy(C) "tails" (approx. 15 residues) were added by terminal deoxynucleotidyl transferase. Plasmid pBR322 DNA was cleaved with PstI endonuclease and treated with terminal deoxynucleotidyl transferase to add deoxy(G) "tails" (approx. 10 residues). The tailed dsDNA and PstI-cleaved plasmid were annealed and used to transform E. coli strain X1776 essentially as described previously (8). Transformed cells were selected on agar plates containing 15 μg/ml tetracycline. The transformed colonies were screened for the presence of L-chain DNA sequences by the Grunstein-Hogness technique (16) using as probe [^{32}P] cDNA of L-321. Plasmids recovered from positive colonies were characterized by digestion with restriction enzymes, and by measuring their capacity to anneal in solution with a highly purified [^{3}H] cDNA prepared from L-321 mRNA (14). All recombinant DNA experiments were done under P3-EK2 containment.

Antibodies. Purified antibodies were used for Ig L-chain identification.

These were isolated from goat antisera to L-321 and rabbit antisera to normal goat-Ig by passage of the antisera on specific immunoadsorbents (11). Rabbit antisera to β-galactosidase was a gift from Dr. Y. Yariv. The purified antibodies and L-321 protein were [^{125}I]-radiolabeled as described (17).

In situ colony radioimmunoassay. All operations were carried out at room temperature unless otherwise stated. Diazobenzyloxymethyl (DBM) filter paper was prepared from the m-nitrobenzyloxymethyl derivative (Miles-Yeda Ltd. Rehovot, Israel) as described (18). The final washings were done in 50 mM Na phosphate pH 8.0 (PB). Bacterial colonies grown on supplemented L broth (8)-agar and exposed to chloroform vapor (19), were transferred to a freshly prepared DBM paper disc by placing the paper on the agar surface and pressing lightly. To accomplish binding, the paper, with the adsorbed colonies facing up, was placed for 2 h over a sheet of filter paper (Whatmann 3 MM) covering a shallow dish filled to the top with PB. This allows diffusion of the buffer from the dish through the paper cover to the diazotized paper disc. The disc was then similarly placed over modified Denhardt's solution (20) containing 0.02% polyvinylsulfate, 0.02% Ficoll, 0.1% BSA, 1% glycine and 50 mM Na phosphate pH 8.0. Following an overnight incubation at 37° (in a closed vessel to minimize evaporation), the filters were washed twice for 10 min periods over PSB (50 mM Na phosphate pH 8.0, 0.15 M NaCl, 0.1% BSA) and then immersed for 2-4 h, at 4°, in 3 ml of a solution containing 5-15 μg/ml of purified goat anti L-321 antibodies in PSB. Unbound antibodies were removed by gently immersing the disc twice for 10 min in PSB containing 0.1% Triton X-100, and twice for 10 min in PSB. Each paper disc was then soaked in 1.0 ml of PSB containing 5-8 x 10^6 cpm (measured in a gamma counter) of [^{125}I]-rabbit anti-goat Ig antibodies (about 10^7 cpm/μg), incubated for 2 h at 4°, and washed by repeating the same washing steps as after the binding of the first antibody, with additional washes in 2 x SSC and finally in PSB. Following air-drying, the discs were exposed to Agfa-Gevaert Curix RP2 film for 1-2 days.

Bacterial extracts. Extracts of spheroplasts and periplasmic space were prepared essentially as described (7), but with a few modifications. Bacteria were grown in 250 ml of supplemented L broth (8) to a density of about 5 x 10^8 cells/ml. Pelleted cells were washed once with 10 mM Tris-HCl, pH 8.0, and resuspended to a final density of approximately 10^{10} cells/ml in a solution containing 20% sucrose, 33 mM Tris-HCl pH 8.0, and 1 mM phenylmethylsulphonyl floride (PMSF). EDTA was added to a final concentration of 1 mM and the mixture was left in ice for 5 min. Lysozyme was then added to 100 μg/ml and incubation in ice was continued for 30 min. The mixture was centrifuged at

10,000 rpm for 10 min in the cold, and the supernatant fraction was considered to include the contents of the periplasmic space. The pellet was resuspended in 1/4 the volume of the previous suspension in a solution containing 10 mM Tris-HCl pH 8.0, 1 mM PMSF, 10 mM $MgCl_2$, 20 µg/ml DNase A and 100 µg/ml RNase A, and further treated as described to prepare the cytoplasmic extract (7). The periplasmic fraction was also prepared by subjecting the cells to osmotic shock, as described (8, 21).

Binding competition-radioimmunoassay in solution. The assays (essentially as described in ref. 7) were carried out in 150 µl mixtures containing 0.07 µg of purified goat anti L-321 antibodies (sufficient to bind about 50% of the iodinated L-321), 3.0 µg of normal goat-Ig, 23,000 cpm of [^{125}I] L-321 (specific activity app. 10^7 cpm/µg), and varying amounts of unlabeled L-321, or bacterial extracts. The mixtures, also containing 0.1 M Na phosphate pH 7.5, 0.15 M NaCl and 5 mg/ml BSA, were incubated for 4 h at 4° and the goat Ig was precipitated by adding 65 µg of purified rabbit anti-goat-Ig antibodies in 150 µl of the above medium, and incubating overnight in the cold. The mixtures were centrifuged for 10 min in the Eppendorf Microfuge and radioactivity in supernatants and pellets was determined in a gamma counter. Nonspecific precipitation of [^{125}I] L-321 protein, determined in the absence of anti L-321 antibodies, amounted to 3-4% of the input.

RESULTS AND DISCUSSION

Identification of L-321 cDNA clones. *E. coli* X1776 cells transformed with pBR322 annealed to L-321 dsDNA were plated on tetracycline (Tc) containing plates. The yield of Tc-resistant transformants was 50-200 colonies per ng of dsDNA in the annealed mixture. About 55% of the Tc-resistant colonies gave a positive reaction by *in situ* hybridization using as probe a highly purified [^{32}P] cDNA of L-321 (14). To ascertain that the *in situ* hybridization scored L-chain DNA, and not unrelated insert DNA that could hybridize with trace contaminants in the [^{32}P] cDNA probe, we tested some recombinant plasmid DNA's for their capacity to anneal and protect highly purified L-321 [^{3}H] cDNA (> 95% pure) from S1 nuclease digestion (22). In all 10 cases tested we found significant protection (over 12%) of the [^{3}H] cDNA by the plasmid DNA, strongly indicating that the cloned DNA insert codes for the κ L-chain. For example (Table 1), the DNA from plasmid B13 was able to protect 28% of the [^{3}H] cDNA from S1 nuclease digestion while the DNA of pBR322 did not hybridize to L-321 [^{3}H] cDNA.

Chimeric plasmids were isolated from 20 positive transformants and the DNA's

TABLE 1. Protection of L-321 [^{3}H] cDNA by plasmid DNA from digestion by S1-nuclease.

UNLABELED DNA	SIZE OF CLONED DNA (bp)	PERCENT OF [^{3}H] cDNA PROTECTED
B13	700	28
B18	680	37
B23	1020	42
pBR322	-	1.1
L-321 m RNA		98
None		0.9

The [^{3}H] cDNA was prepared from a highly purified L-321 mRNA by AMV reverse transcriptase. Hybridization was carried out in 50 µl containing 2 µg of plasmid DNA, 2 ng (3000 cpm) of [^{3}H] cDNA, 0.3 M NaCl, 10 mM Tris-HCl, pH 7.4, 2 mM EDTA and 0.1% $NaDodso_4$. In one control the plasmid DNA was replaced by 60 ng of L-321 mRNA. Prior to hybridization plasmid DNAs were digested with Hae III, followed by boiling for 2 minutes. Hybridization was performed at 68° for 2 hrs. Hybrids were assayed by treatment with S1-nuclease (22). B13, B18 and B23 are recombinant pBR322 plasmids containing κ L-321 sequences.

were cleaved with PstI. Gel-electrophoretic analyses (not shown) indicated the cloned DNA inserts ranged in size between 250-850 bp. Four plasmids yielded two PstI fragments derived from the insert: one invariable fragment of 850 bp and another smaller fragment of variable length extending up to 170 bp. From these results it was inferred that the longest cloned DNA insert was about 1020 bp, and that it contained one PstI site in the variable region coding sequence located about 850 bases from the 3' and of the dsDNA (orientation corresponding to mRNA).

Expression of Ig L-chain antigenic determinants in bacterial clones. A new method has been devised to screen for the presence of specific antigenic determinants in bacterial colonies. The method, described in detail in the Materials and Methods section, employs diazonium derivatized filter paper as a solid support for proteins of bacterial colonies. Colonies lysed by chloroform are transferred to the paper and incubated under conditions shown in preliminary trials to allow efficient coupling of tyrosine to the paper. Conceivably, these conditions are also suitable for the covalent binding of the cellular proteins to the solid support. After the initial binding step the paper is soaked in a solution containing bovine serum albumin at pH 8.0 in order to inactivate unreacted diazonium groups. This is followed by exposure

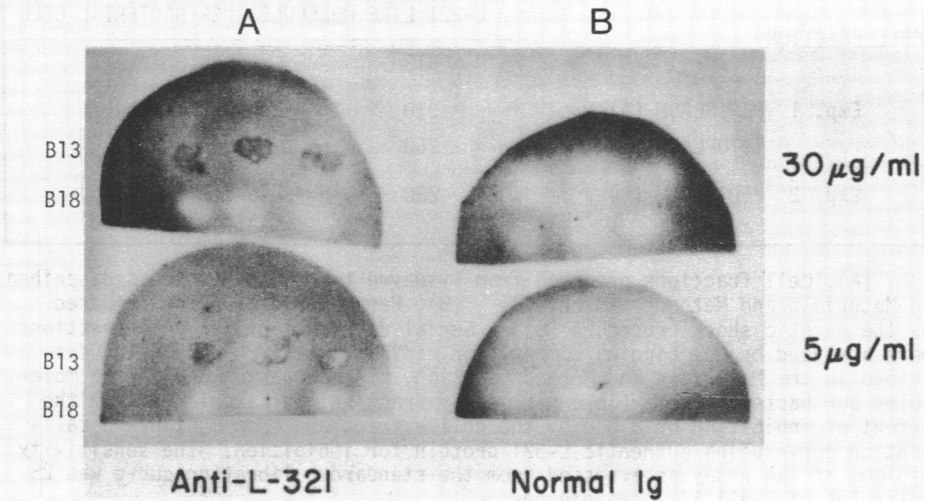
to antibodies against the antigen under study, i.e., purified goat anti L-321 antibodies. Following intervening washing steps, the filters are treated with ^{125}I-labeled antibodies against the first antibodies (rabbit anti-goat-Ig antibodies), washed and autoradiographed.

The specificity of the method was demonstrated by comparing an E. coli mutant constitutive for β-galactosidase synthesis with a β-galactosidase deletion mutant, using for the assay β-galactosidase immune serum from rabbit, normal rabbit serum, and [^{125}I] goat anti-rabbit Ig antibodies (data not shown). A clear positive reaction was evidenced only in colonies of the bacteria synthesizing β-galactosidase and treated with anti β-galactosidase antibodies, thus verifying the specificity of the assay. The major advantage of the in situ double antibody method lies in its ability to score for the expression of different proteins by using a single radiolabeled antibody preparation. For example, specific, unlabeled rabbit antibodies to proteins A, B or C can be applied in the first stage. Colonies expressing any of these proteins will be scored by the same ^{125}I-labeled antibodies against rabbit Ig; however, the method can not detect the expression of fused polypeptides in a single assay (cf. ref. 19).

Seven different independently isolated bacterial clones with plasmids containing L-321 DNA inserts ranging in length from 430 to 1020 base pairs were screened for L-321 antigenic determinants by the method described. One of the tested clones - B13 - gave a clear positive response. The specificity of this response is demonstrated in analyses of the B13 clone together with the negative B18 clone (Fig. 1), in which the two clones were exposed to two concentrations of anti L-321 antibodies, or normal goat Ig. Positive reactions are seen only in colonies of clone B13 treated with anti L-321 antibodies, the intensity of the reaction increasing somewhat with antibody concentration (Fig. 1).

The cloning of L-321 DNA within the β-lactamase gene may result in the synthesis of a chimeric polypeptide where the L-321 sequence forms an extension of the N-terminal part of β-lactamase. Such a fused polypeptide may be transported across the cell membrane by virtue of the N-terminal sequence of β-lactamase (23, 24) as has been first observed in a clone synthesizing pre-proinsulin (8). To test this possibility cells of clone B13 were fractionated into periplasmic and cytoplasmic fractions and each fraction was analyzed for the presence of L-321 like peptide by its ability to inhibit the binding of authentic [^{125}I] L-321 to anti L-321 antibodies. Analogous fractions from clone B18, negative in the in situ radioimmunoassay (Fig. 1), were tested as a

Figure 1. Identification of L-321 protein sequence in bacteria by in situ colony radioimmunoassay.



Colonies of clones B13 and B18 carrying recombinant plasmids including an insert of L-321 cDNA (see Table 1) were transferred to DBM paper discs and assayed according to the procedure described in Materials and Methods using anti L-321 antibodies or normal goat Ig at the concentrations indicated.

control. The results showed that both types of periplasmic preparation as well as the cytoplasmic fraction of clone B13 were effective in inhibiting the binding of L-321 while fractions of clone B18 were ineffective.

A quantitative estimate based on the number of cells extracted, the inhibitory capacity of the extracts and comparison with a standard radioimmunoassay curve indicates (Table 2) an approximately equal distribution of L-321 like polypeptides in both cellular compartments with about 300 molecules in each compartment per cell (Table 2). Determination of total protein contents by the Lowry procedure (25) indicated that the cytoplasmic fraction contained 8-10 fold more protein than the corresponding periplasmic fraction. Considering this ratio and the presence of comparable amounts of L-321 like protein in the two cellular compartments, it appears that the L-321 like molecules are preferentially segregated into the periplasmic space.

These results make it likely that the L-321 like polypeptide is transported across the cell membrane, and hence that the Ig sequence is synthesized contiguously with the N-terminal portion of β-lactamase forming a single hybrid polypeptide. The hydrophobic amino-terminal extra piece of L-321 normally involved in secretion of Ig proteins (27) cannot be responsible for the observed transport since its coding sequence is not present in the cloned

TABLE 2. Immunoreactive L-321 protein in bacterial extracts.

CELL FRACTION		L-321 LIKE MOLECULES PER BACTERIAL CELL	
		B13	B18
Exp. 1	PERIPLASM (A)	310	Undetectable
	CYTOPLASM (A)	260	Undetectable
Exp. 2	PERIPLASM (B)	280	Undetectable

(A), Cell fractions prepared from lysozyme treated bacteria as described in Materials and Methods and ref. 7. (B), Periplasmic fraction prepared by the osmotic shock procedure (8). Serial dilutions of the cell fractions were analyzed by the binding competition radioimmunoassay in solution described in the Materials and Methods section. The number of L-321 like molecules per bacterial cell (in a particular fraction) is calculated from the extent of inhibition obtained by the cell extract and from a standard calibration curve using authentic L-321 protein for inhibition. The sensitivity minimum of the assay as assessed from the standard calibration curve was 25 molecules of L-321 like protein per cell.

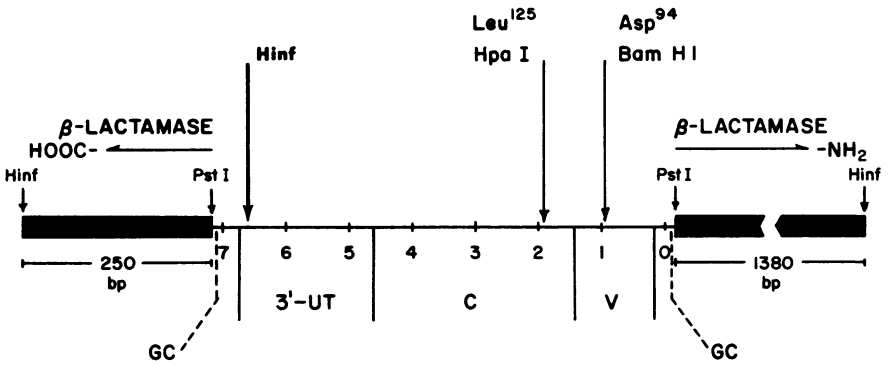
section of the cDNA (see below). The formation of a fused polypeptide of the type described is possible provided the cDNA is inserted in the PstI site of the cloning vehicle in the proper orientation and in phase with respect to the β-lactamase gene.

Orientation of L-321 DNA cloned in B13 plasmid. The B13 plasmid was digested with several restriction enzymes, and the gel electrophoretic patterns obtained enabled the construction of the map shown in Figure 2. The insert corresponds to only part of the V region (coding for about 40 amino acids), the entire C region, and the 3' untranslated region of the mRNA. The partial V region sequence is continuous with the amino terminal coding region of the β-lactamase gene (Fig. 2). That is, in B13 the cloned L-321 DNA has the same orientation as the β-lactamase gene. Restriction analysis of clone B18 (data not shown) showed that in this plasmid the L-321 DNA was aligned in the reverse orientation as compared to B13. That is, in B18 the Hinf site close to the 3' end of the cDNA is adjacent to the Hinf site of pBR322 located 1380 bp away from the PstI site (cf. Figure 2). This orientation cannot lead to the formation of a chimeric protein composed of β-lactamase and L-chain polypeptide.

Concluding remarks. The PstI site in the β-lactamase gene of pBR322 was used for insertion of Ig L-321 DNA sequences. One of the recombinant plasmids, B13, was capable of directing in bacteria the synthesis of an immunoglobulin L-chain like protein.

The immunological characterization of the L-321 like protein employed two

Figure 2. Restriction enzyme cleavage map of the L-321 cDNA insert in the recombinant plasmid isolated from clone B13.



The insert is represented by a line and flanking pBR322 sequences as dark bars. The scale refers to bp · 10^{-2}. Restriction sites were determined by the gel electrophoretic analysis of single and double enzyme digestions. The scheme also shows the boundaries of the parts corresponding to the V (variable) C (constant) and 3' UT (3' untranslated) regions of the mRNA; as well as the oligo (dG): oligo (dC) stretches introduced by the tailing step. The alignment of restriction sites with defined positions was based on the following: the published nucleotide sequence of the C and 3' UT regions of mouse κ L chain DNA (27) localize a Hinf site 11 nucleotides from the coded 3' end of the 3'-UT region, and a HpaI site at the codon for Leu^{125} in the C region. Restriction mapping and computer analysis of L-321 DNA predict a Bam HI site at the codon for Asp^{94} in the V region (28), as recently confirmed by the nucleotide sequence of this region (29).

Adjacent Hinf sites on pBR322 DNA located 1380 and 250 bp from ends of the insert served to orient the cloned cDNA with respect to the DNA encoding the amino and carboxyl termini of β-lactamase.

types of assay: a newly developed in situ colony radioimmunoassay and a binding competition assay. In both types of assay colonies or cellular extracts of the B13 clone, but not of other clones tested, reacted with anti L-321 antibodies and failed to react with control normal Ig.

The evidence for the membrane transport of the L-321 like material combined with the orientation of the cloned L-321 cDNA with respect to the β-lactamase gene strongly suggest that the L-321 sequence is synthesized by the same mechanism as first reported for the expression of rat preproinsulin in bacteria (8). That is, the immunoglobulin synthesis occurs by a readthrough mechanism utilizing both transcriptional and translational initiation signals of the β-lactamase gene and resulting in the formation of a chimeric polypeptide consisting of the N-terminal portion of β-lactamase followed by several glycine residues and the L-321 sequence (cf. 8).

It is noteworthy that the B13 clone expressing L-321 antigenic determinants has been found in a screen of a total of seven independently isolated

clones of L-321 cDNA. A random frequency of one positive clone out of six is to be expected for this mode of cloning if one considers the orientation and the reading frame of the inserted cDNA with respect to the plasmid DNA as the only factors determining its expression as a readthrough product.

The structure of the B13 plasmid (Fig. 2) shows that the L-321 DNA can direct the synthesis of only part of the L-chain; about 40 amino acids of the V region and the entire C region. It lacks the information for the initiator methionine, the N-terminal short lived hydrophobic extra piece (26), as well as for approximately 70 amino acids of the mature V region. Despite these abberations and the fusion to the β-lactamase peptide, it appears that at least the C region can fold to generate antigenic determinants recognizable by antibodies to the L chain. Evidently, this reflects the capacity of immunoglobulin chains to fold into distinct C and V regions (30).

Altogether, these results indicate the possibility of the synthesis of specific antibody molecules in bacteria.

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